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## Anti-inflammation and anti-platelet aggregation activities of the ethanolic extract of *Graptophyllum pictum* leaves in wistar rats

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### ABSTRACT

*Graptophyllum pictum* is a well-known folk medicine in Indonesia. The red leaves are widely used for their anti-inflammatory properties to relieve hemorrhoids. However, little is known about to what extent these properties can affect platelet aggregation. This study was aimed to determine the anti-inflammation and anti-platelet aggregation activities of the ethanolic extract of *Graptophyllum pictum* (EEGP). Apart from the ear and hind-paw edema assay, anti-platelet aggregation and bleeding time assay were also performed on male Wistar rats. The test animals were grouped into six groups: three treatment groups termed EEGP100, EEGP600, and EEGP3000 (received 100, 600, and 3000 mg/kg BW of EEGP, respectively), one control-solvent group (1% CMC-Na), and two positive control groups (aspirin and Na-diclofenac). Based on the percentage of edema inhibition, absorbance change, and bleeding time, EEGP600 and EEGP3000 produced a comparable percentage of edema inhibition to that of Na-diclofenac, which was not found in EEGP100. Also, EEGP600 and EEGP3000, but not EEGP100, inhibited platelet aggregation as effective as aspirin (positive control). In conclusion, if administered at 600 and 3000 mg/kg BW, the ethanolic extract of *Graptophyllum pictum* can function as an anti-inflammatory agent that inhibits platelet aggregation.

**Keywords:** anti-inflammation, anti-platelet aggregation, *Graptophyllum pictum*

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## INTRODUCTION

*Graptophyllum pictum* (synonym: *Justicia picta*) is a popular folk medicine in many countries, including Indonesia. For three decades, the ethanol extraction of its red leaves has been known to exhibit anti-inflammatory activities (Shah, 2011), a precursor of hemorrhoid treatments. Prior scholars worldwide have been extensively reporting the myriad benefits of *G. pictum* (Goun et al., 2003; Olagbende-Dada et al., 2011; Srinivasan et al., 2011; Jiangseubchatveera et al., 2015; Jiangseubchatveera et al., 2017). Nevertheless, little to no research has revealed whether or not, and to what extent, the anti-inflammatory properties of *G. pictum* affect platelet aggregation.

Hemorrhoid is a condition where an anal sphincter, a group of muscles, is hypertrophic. It is also believed to occur because of the varicose dilatation of the anal veins (Loder, 1994; Patil et al., 2019). Aside from varicose dilatation, venous thrombosis is also a common trigger of internal hemorrhoids (Loder, 1994; Lohsiriwat, 2012; Patil et al., 2019). For this reason, the pharmacological treatment of these conditions mostly targets the reduction of vascular inflammation and thrombosis (Lohsiriwat, 2012; Sya'haya, 2016). Inflammation triggers the release of inflammatory mediators, such as histamine, serotonin, prostaglandin, and thromboxane A<sub>2</sub>, in the cyclooxygenase pathway (Capurso and Capurso, 2020). All of these mediators has arachidonic acid as the precursor. Thromboxane synthase converts arachidonic acid to prostaglandin H<sub>2</sub> (PGH<sub>2</sub>), which then transforms into thromboxane A<sub>2</sub> that later activates platelet aggregation (Capurso and Capurso, 2020). Therefore, the inhibition of inflammation will be followed by impeded platelet aggregation.

This research reported the anti-inflammatory activity of EEGP—which can trigger the inhibition of platelet aggregation—as an attempt to explore the development of EEGP for hemorrhoids treatment.

## MATERIALS AND METHOD

### Materials

The materials used in this research were red leaves of *Graptophyllum pictum*, carrageenan, arachidonic acid (Sigma), aspirin (Phapros), and Na-diclofenac (Phapros). *G. pictum* leaves were collected from Tegal, Jawa Tengah Province, Indonesia, and authenticated by Mr. Arif Husin, M.Si., the plant taxonomist at Botany Laboratory, Muhammadiyah University of Purwokerto.

### Animals

The experiment used male Wistar rats aged 2-3 months old and weighing 150-250 g and allowed them to first acclimatize to the new surroundings for seven days. Before serial anti-inflammation assay, these test animals were given EEGP at doses of 100, 600, and 3000 mg/kg BW for seven days. They received a normal diet and oral administration of the test drugs and extracts.

### Methods

#### Preparation of ethanolic extract of *Graptophyllum pictum* (EEGP)

*G. pictum* leaves were washed under running water, dried underneath a black cloth to prevent direct sunlight, and grounded into powder. Then, the powder was macerated using 96% ethanol in a 1:10 ratio for 24 hours and mixed every 6 hours for 24 hours. The macerate collected from the first process was processed with another cycle of maceration using half volume of the solvent. All the macerates from the first and second cycles were then mixed and evaporated using a vacuum evaporator to obtain a viscous extract.

#### The grouping of test animals

The test animals were clustered into four groups of treatment. Group I was a control-solvent group administered with 1% Na-CMC for seven days. Group II was a positive control group for the edema assay that was given Na-diclofenac at 12.6 mg/kg BW two hours prior to the assay. Group III was a positive control group for anti-platelet aggregation that received aspirin at 10.1 mg/kg BW.

Groups IV, V, and VI were treatment groups receiving 100, 600, and 3000 mg/kg BW of EEGP, each of which denoted as EEGP100, EEGP600, and EEGP3000. All steps of the experiment were performed ethically to avoid animals from suffering, and at the final step, all animals were sacrificed by cervical dislocation. These experiment procedures had been assessed by the Research Ethics Committee of the Muhammadiyah University of Purwokerto with the ethical approval document number KEPK/UMP/23/VI/2020.

### Ear edema test

This assay followed the procedures described in [Dunstan \*et al.\* \(1997\)](#). The ear thickness of all groups was measured before and after the induction of edema. A total of 0.01 ml of arachidonic acid (0.58 mM in 0.9% NaCl) was applied to the rat ears, and then the ear thicknesses were measured after 30, 60, 90, 120, 180, and 240 minutes. The percentage of ear edema in each group was calculated using the formula below:

$$\% \text{ edema} = \frac{Tt - T0}{T0} \times 100\%$$

where  $Tt$ = ear thickness after arachidonic acid injection, and  $T0$ = ear thickness before arachidonic acid application. Then, the percentage of edema inhibition was computed as follows:

$$\% \text{ edema inhibition} = \frac{a - b}{a} \times 100\%$$

where  $a$ = % edema of the control-solvent group, and  $b$ = % edema of the treatment groups.

### Hind-paw edema assay

The assay was performed using plethysmography, a testing method created and introduced by Winter and colleagues decades ago but still extensively used until today ([Rege \*et al.\*, 2020](#)). The hind paws were dipped into an apparatus containing mercury, then the number showing the mercury volume being increased due to the immersion was documented. This number represents the volume of the hind paw before the treatment ( $V_0$ ). After the sub-plantar injection of 0.3 mL of 1% carrageenan, the volume of the induced edema was documented as volume after treatment ( $V_t$ ) at Minute 30, 60, 90, 120, 180, 250, 300, and 360. The percentage of hind paw edema was then calculated using the formula below:

$$\% \text{ edema} = \frac{Vt - V0}{V0} \times 100\%$$

where  $Tt$ = volume of hind paw edema after carrageenan injection, and  $T0$ = volume of hind paw before carrageenan injection. Then, the percentage of edema inhibition was calculated as follows:

$$\% \text{ edema inhibition} = \frac{a - b}{a} \times 100\%$$

where  $a$ = % edema of the control-solvent group, and  $b$  = % edema of the treatment groups.

### Anti-platelet aggregation assay

Blood was drawn from the tails, added with 1% EDTA, mixed to prevent clotting, and centrifuged at 6,000 rpm for 3 minutes. A total of 293.5  $\mu$ l of supernatant was then collected and

added with 6.5  $\mu$ l of arachidonic acid to create a final concentration of 12.38 mM. The absorbances of the solution were measured at a wavelength of 655 nm using a microplate reader (iMark, Biorad) 15 seconds, 30 seconds, and minute for 10 minutes (Sukandar, 2008).

### Tail bleeding time assay

Bleeding time assay was performed according to the procedures described in Min et al. (2012). After cutting off the tail tip, the bleeding area was dabbed with a filter paper (Whatman) every 30 seconds until the bleeding stopped (no apparent bloodstain on the paper). The time lapsed from tail tip amputation to cessation of bleeding was recorded as bleeding time.

### Data Analysis

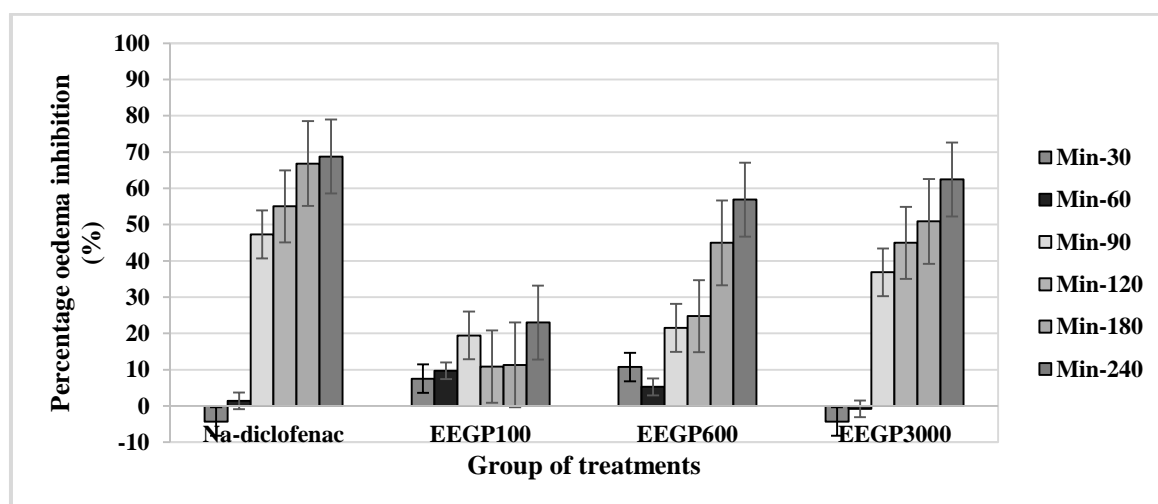
Percent edema inhibition (obtained from the ear and hind paw edema assay), changes in absorbance values (anti-platelet aggregation assay), and bleeding time were analyzed using two-way Analysis of Variance (ANOVA) in SPSS v.16.0 program.

## RESULTS AND DISCUSSION

### EEGP exhibits anti-inflammation activity by reducing edema in rat ears and hind paws

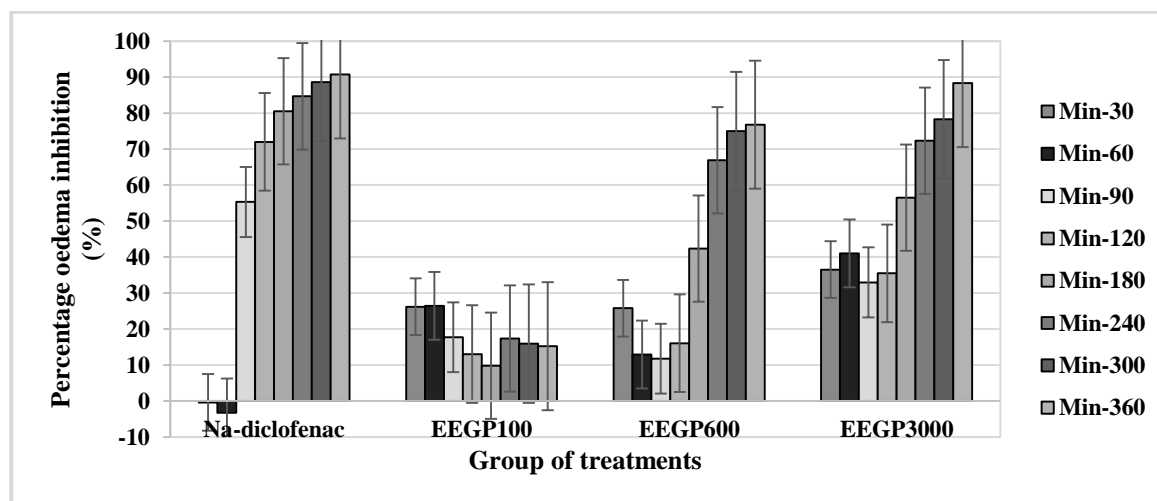
The anti-inflammatory properties of EEGP, as reported in Shah et al. (2011), was confirmed by the ear and hind paw edema assay. Ear edema is a symptom of inflammation and, in this study, was measured subsequent to the application of arachidonic acid, an edema inducer. Changes or swelling in ear thickness were observed by measuring the thickness of the pinna (auricle) using calipers. Percent edema represents the difference between ear thicknesses before and after its induction, while % edema inhibition for each treatment group is expressed as the normalized value against the control-solvent group (Figure 1).

Figure 1 shows the percentage of ear edema inhibition from different treatment groups. Na-diclofenac and EEGP at all concentrations inhibited ear edema 90 minutes after the arachidonic acid injection. Na-diclofenac, as a positive control for edema inhibition, showed a larger percentage of inhibition compared to all concentrations of EEGP. EEGP100 could not inhibit the ear edema even after Minute 240, whereas EEGP600 and EEGP3000 exhibited inhibitory activities comparable to that of Na-diclofenac—as evident from  $p < 0.05$  (statistically non-significant difference).



**Figure 1. Percentage of edema inhibition in the ear edema assay. The percentage is the mean  $\pm$  SE values of triplicate experiments**

The hind paw edema assay was performed to support the results of the ear edema assay, which examined the anti-inflammatory activities of EEGP. Percent of carrageenan-induced edema in hind paws represents the difference between the volumes of edema before and after its induction, as observed using the plethysmograph instrument. Whether or not the drugs and EEGP could reduce these volumes, it is reflected by % edema inhibition of each treatment group, which is a normalized value against the control-solvent group (Figure 2.).



**Figure 2. Percentage of edema inhibition in the hind paw edema assay. The percentage is the mean  $\pm$  SE values of triplicate experiments**

Figure 2 displays the percentage of hind paw edema inhibition in different treatment groups. Na-diclofenac, as a positive control, was found to inhibit hind paw edema in Minute 90 and exhibit increased inhibitory activities by up to 90% in Minute 360. EEGP100 did not impede this edema even after 360 minutes had passed from the carrageenan injection. Both EEGP600 and EEGP3000 exhibited signs of hind paw edema inhibition, which were not significantly different from the outcome of Na-diclofenac ( $p < 0.05$ ). EEGP600 had the same % edema inhibition (i.e., ~40%) as EEGP3000 in Minute 180, which was 150 minutes later than EEGP3000. Nevertheless, no variety of % edema inhibition was apparent in a more extended period (Minute 240 to 360).

The ear and hind paw edema assay revealed that unlike EEGP100, both EEGP600 and EEGP3000 could impede the progression and reduce edema. In addition, there was no statistical difference among the % inhibitions in the ear and hind paw edema assay ( $p < 0.05$ ).

### EEGP inhibits platelet aggregation

Arachidonic acid, as the platelet aggregation-triggering agent, binds to a platelet membrane receptor and, subsequently, activates the platelet to release its granule content, resulting in an increased rate of aggregation. Platelet activation or aggregation was observed based on the turbidimetry principle, i.e., from periodic changes in plasma absorbance at a wavelength of 655 nm (Sukandar, 2008). After the injection of arachidonic acid, plasma absorbance lowers as the platelet starts to form aggregates, reducing the turbidity of the plasma (Sukandar, 2008). Low plasma absorbance appears when platelet aggregation is inhibited. As seen in Table 1, EEGP100 showed immense changes changes in absorbance. Meanwhile, EEGP600 produced similar changes in absorbance as aspirin—a positive control in the experiment—and EEGP3000 showed smaller absorbance changes compared to aspirin. The absorbance changes of EEGP600 and EEGP3000 groups were not significantly different from the ones produced by aspirin, as evident from the statistical analysis results ( $p < 0.05$ ).

**Table 1. Platelet aggregation profiles after the administration of different concentrations of *Graptophyllum pictum* leaf extracts, as measured by absorbance changes in different points of time (0.15, 5, and 10 minutes)**

Treatment Groups	Minutes 0.15	Minutes 5	Minutes 10	$\Delta$ 0.15-5	$\Delta$ 0.15-10
EEGP100	0.650 $\pm$ 0.053	0.512 $\pm$ 0.183	0.462 $\pm$ 0.318	0.138	0.189
EEGP600	0.373 $\pm$ 0.057	0.347 $\pm$ 0.093	0.332 $\pm$ 0.112	0.026	0.041
EEGP3000	0.209 $\pm$ 0.112	0.207 $\pm$ 0.116	0.212 $\pm$ 0.111	0.002	-0.003
Solvent-control	0.522 $\pm$ 0.061	0.341 $\pm$ 0.051	0.387 $\pm$ 0.057	0.181	0.225
Aspirin (positive control)	0.663 $\pm$ 0.053	0.639 $\pm$ 0.045	0.803 $\pm$ 0.048	0.024	0.020

Note: Absorbance values are presented as mean  $\pm$  SD of triplicate experiments. Minus (-) indicates an increase in absorbance. The absorbance changes ( $\Delta$ ) is the difference between the absorbance subtracted in Minute 0.15 and the absorbance of the respective time.

Bleeding time assay investigated the effects of EEGP, aspirin, and Na-diclofenac on hemostatic mechanism. In this experiment, anti-platelet aggregation is indicated by a longer bleeding time after the administration of EEGP. As seen in Table 2, all treatment groups required a more extended time to stop the bleeding after the tail tip amputation. Similar to the outcome of the ear and hind paw edema assay and anti-platelet aggregation assay, EEGP600 and EEGP3000 tended to exhibit the same effect as Na-diclofenac and aspirin, respectively. The statistical analysis revealed that the bleeding time of test rats receiving EEGP600 was not significantly different from the ones given Na-diclofenac ( $p < 0.05$ ). Comparable results were also identified between the EEGP3000 group and the positive control group, i.e., aspirin ( $p < 0.05$ ).

**Table 2. The bleeding time after tail tip amputation in rats receiving *Graptophyllum pictum* leaf extracts, Na-diclofenac and aspirin (positive controls), and solvent (control)**

Treatment Groups	Bleeding time (in seconds)
Solvent-control	100 $\pm$ 17.3
Positive control (Na-diclofenac)	200 $\pm$ 17.3
Positive control (Aspirin)	220 $\pm$ 45.8
EEGP100	120 $\pm$ 0
EEGP600	170 $\pm$ 17.3
EEGP3000	260 $\pm$ 17.3

Note: The bleeding time is the time elapsed between tail tip amputation and cessation of bleeding (no apparent bloodstain on the tissue paper) and is expressed as the mean  $\pm$  SD of triplicate experiments.

Except for EEGP100, both EEGP600 and EEGP3000 have been confirmed to possess anti-inflammatory properties that are likely to inhibit platelet aggregation. This effect, indicated by a longer bleeding time, emerges due to the inhibited release of thromboxane A2 (Capurso and Capurso, 2020). However, it was contradictory to Tantio (2008), which reported that at 0.018mL/gram BW (or ~100 mg/kg BW), *Graptophyllum pictum* results in a shorter bleeding time than the solvent-control group. Unfortunately, a direct comparison with Tantio (2008) is out of the question because this previous



study only used one dose, i.e., 100 mg/kg BW. Even though, the same single dose has been proven to exhibit the effects of phlebotropic drug on hemorrhoids in a Wistar rat model (Hutagalung, 2019).

Bleeding can occur from the hemorrhoids process due to damages to hemorrhoid surfaces (Loder, 1994; Patil *et al.*, 2019; Zeinalinejad *et al.*, 2019). Accordingly, hemorrhoid treatments are also targeted at stopping or preventing anal bleeding in addition to inhibiting inflammation (Patil *et al.*, 2019). However, the reduction of edema by anti-inflammatory agents can cause bleeding since the platelet aggregation is inhibited following the impeded release of thromboxane A2 (Capurso and Capurso, 2020). As confirmed in this current study, EEGP exhibited anti-inflammatory activities that induced the inhibition of platelet aggregation. Consequently, the development of *G. pictum* for anti-hemorrhoid based on its anti-inflammatory properties must factor in bleeding, as a potential side effect. In Astana (2017), when combined with *Desmodium triquetrum* leaves, *Coleus atropurpureus* leaves, *Curcuma domestica* rhizome, *Curcuma xanthorrhiza* rhizome, and *Phyllanthus niruri* herbs, 30% *G. pictum* leaves are reportedly safe to use. However, at which range of dose *G. pictum* can exhibit anti-inflammatory activity without causing bleeding needs to be studied.

## CONCLUSION

The ethanolic extract of *Graptophyllum pictum* leaves at doses of 600 and 3000 mg/kg BW exhibit anti-inflammatory activities against arachidonic acid-induced ear edema and carrageenan-induced hind paw edema in Wistar rats, as evident from the inhibition of platelet aggregation. Therefore, it can be further developed as a potential treatment for hemorrhoids.

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